Supporting Information

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SI Materials and Methods

Antibodies and Reagents. The following antibodies were used: rabbit antiserum to human Clq (Dako); goat antiserum to human C3, C4, C1s (Quiidel); rabbit anti-human C1r (Sigma–Aldrich); mouse monoclonal anti-Ki67 (clone MIB1; Dako) and anti-CD34 (NovocastraOBEEnd/10); rabbit anti-gC1q receptor (gC1qR) and anti-cC1q receptor (cC1qR) and their F(ab')2 fragment (1); rabbit IgG anti-NG2 (Chemicon International); sheep alkaline phosphatase-conjugated IgG antidigoxigenin (Roche); TRITC-labeled goat anti-rabbit IgG and HRP-conjugated anti-mouse IgG (Dako); and alkaline phosphatase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG (Sigma–Aldrich). Purified Clq, VEGF, bradykinin, and polymyxin B were from Sigma–Aldrich. Clq was also purified from fresh human serum according to Stemmer and Loos (2) and used to confirm the in vitro data obtained with the commercial C1q used in all of the in vitro and in vivo experiments. Clq was digested with type III collagenase [6.6% (wt/vol)] or pepsin [4% (wt/vol)] (Sigma–Aldrich) for 20 h at 37 °C to obtain gC1q and cC1q fragments, respectively, and further purified by Resourse S Column (Pharmacia). The purity of Clq and C1q fragments was checked by SDS/PAGE analysis. Cls and C1r were partially purified by the method of Stroud and Sakai (3) and C1s-deficient serum was obtained from a patient identified by F.T.

Human Tissues. Dermal granulation tissue samples (n = 3) were obtained from the archives of the Department of Human Pathology, University of Palermo, Italy. Skin biopsy specimens from three patients with skin ulcers followed at Trieste University Hospital, Italy were used for in situ hybridization and quantitative real-time PCR (qPCR). The study was approved by the Institutional Board of Trieste University Hospital, Italy and informed consent was obtained from patients providing the tissue specimens.

Immunohistochemical Staining. Four-micrometer-thick paraffin tissue sections were stained for immunohistochemical analysis as previously reported (4) using either the streptavidin–biotin–peroxidase complex method with 3-3‘ diaminobenzidine (DAB) and aminoethyl carbazole as chromogens or the alkaline phosphatase–anti-alkaline phosphatase method and the Fast Red chromogen (Dako).

qPCR. Total RNA was extracted from tissues harvested in Eurogold (Euroclone) and reverse-transcribed as previously described (4). qPCR was carried out on Rotor-Gene 6000 (Corbett Life Science) using iQ TM SYBR Green Supermix (Bio-Rad). The relative amount of the three chains of Clq were normalized with 18S expression and determined using the Rotor Gene 1.7 software (5). RNA expression extracted from blood-derived macrophages was used as calibrator.

In Situ Hybridization. PCR products for the C chain of Clq (Table S1) were used as template for subsequent synthesis of cRNA probes with DIG RNA Labeling Kit (Roche). Frozen sections (7 μm) were treated with protease K (Euroclone) before overnight incubation at 55 °C with digoxigenin-labeled antisense or sense RNA probes that were revealed by alkaline phosphatase – anti-digoxigenin antibody followed by Nitro blue tetrazolium and 5-Bromo-4-chloro 3-indolyl phosphate (Sigma–Aldrich).

ELISA on Human Umbilical Vein Endothelial Cells. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously published (6). Cells were grown to confluence in 96-well tissue culture plates (Nunc; Mascia Brunelli) and incubated with increasing concentrations of purified C1q to a final volume of 100 μL of medium for 1 h at room temperature. Bound C1q was revealed by ELISA using mAb anti-human C1q (10 μL/mL) (Quiidel) and alkaline phosphatase-conjugated secondary antibodies (Sigma–Aldrich). The enzymatic reaction was developed with p-nitrophenyl phosphate (Sigma–Aldrich; 1 mg/mL) as substrate and read kinetically at 405 nm using a TiterTec Multiskan ELISA reader (Flow Labs).

Leakage Assay. The assay was performed following a procedure previously described (7). Briefly, 2 × 105 cells were seeded onto the polycarbonate insert of a 24-well Transwell system (Costar, 6.5-mm diameter, 3-μm diameter pores) coated with 2% gelatin and used 5 d after plating. The stimuli were added to the upper chamber together with FITC-BSA (1 mg/mL), and the fluorescence intensity in the lower chamber was evaluated after 30 min by Infinite200 (Tecan).

Proliferation Assay. HUVECs (105/well) seeded on a 2% gelatin-coated 24-well plate were stimulated with Clq (10 μg/mL) or VEGF (20 ng/mL). After washing, the cells were detached from the wells with trypsin/EDTA (Sigma–Aldrich), seeded on a slide, and fixed first with 4% paraformaldehyde (Sigma–Aldrich) for 1 h and then with cold methanol for 15 min. After two runs in microwaves in Tris/EDTA solution and washings with PBS, the cells were incubated with the antibody to Ki-67 followed by the secondary HRP-conjugated antibody. The enzymatic reaction was developed by adding DAB as substrate (Dako). The number of positive cells was counted in five fields with the highest cellular density under a Leica DM2000 optical microscope.

Migration Assay. Cell migration assay was evaluated by Transwell migration assay, coating the lower side of the polycarbonate filter (8 μm pores) with fibronectin (5 μg/cm2; Roche). HUVECs (2 × 105 cells) in serum-free medium (Invitrogen) were added to the upper compartment and allowed to migrate to the lower compartment filled with Clq (10 μg/mL), VEGF (20 ng/mL), or VEGF (20 ng/mL), or VEGF (20 ng/mL) or VEGF (20 ng/mL) to allow tube formation. After fixation with 4% paraformaldehyde and staining with Phalloidin Alexa Fluor 546 (Invitrogen) the number of tubules was counted under a Leica DM6500 microscope using LAS software (Leica).

Scatch Assay. Confluent monolayers of HUVECs starved for 12 h in serum-free medium were scraped with a pipette tip and, after washing twice with PBS, were incubated with serum-free medium supplemented with Clq (10 μg/mL) or VEGF (20 ng/mL) or medium alone as a control. Wound closure was evaluated up to 24 h under a Leica DMIL inverted microscope (Leica Microsystem) and images were collected using a Canon Powershot A640 digital camera (Canon).

In Vitro Tube Formation. HUVECs (5.5 × 104) placed on wells coated with Matrigel (12 mg/mL) (Becton, Dickinson) were incubated for 24 h with Clq (10 μg/mL) or VEGF (20 ng/mL) to allow tube formation. After fixation with 4% paraformaldehyde and staining with Phalloidin–Alexa Fluor 546 (Invitrogen) the number of tube was counted under a Leica AF6500 microscope using LAS software (Leica).

Immunoblotting. Cells were lysed with RIPA buffer (Sigma–Aldrich) in the presence of the protease inhibitor mixture and the phosphatase inhibitor mixture 2 (Sigma–Aldrich). Proteins were
separated by 12% SDS/PAGE and analyzed by Western blot with the following antibodies: antibody specific for p38 MAPK phosphorylated at Thr180/Tyr182 (3D7), Erk1/2 phosphorylated at Thr202/Tyr204 (197G2), p38 MAPK, and Erk1/2 (137F5). All antibodies were from Cell Signaling Technologies. The intensity of the bands in the Western blot was evaluated by densitometric analysis of Western blot using ImageJ software.

**Rat Aortic Ring Assay.** Rat aortic rings were cultured in a 3D collagen gel for 9 d in the presence of either C1q (10 μg/mL) or VEGF (20 ng/mL). Sprouting formation from aortic rings was evaluated using a Leica DMIL inverted microscope and photomicrographs were taken after 6 and 9 d of incubation with the stimuli. The number and maximal length of vessels and the number of branchings were quantified by the software (Sun SPARC30 and Visilong 5.0) as described by Blacher et al. (8).

To document the presence of endothelial cells (ECs) and pericytes in microvessel sproutings the aortic rings were treated according to Nicosia et al. (9). Briefly, the rings were fixed with 10% formalin (Sigma–Aldrich) and incubated first with 2% BSA (Sigma–Aldrich) in dPBS-0.1% Tween 20 (Sigma–Aldrich) for 1 h at room temperature and then with rabbit IgG anti-NG2 for 1 h followed by the secondary antibody to rabbit IgG for 45 min at room temperature. ECs were stained with biotin-labeled *Griffonia simplicifolia* isoelectin-B4 (Sigma–Aldrich) and FITC-conjugated streptavidin (Dako). The rings were analyzed by the LEICA SP2 confocal laser scanning microscope (Leica Microsystems) using Leica Confocal software.

**Wound Healing in Mice and Rats.** Three groups of C57BL/6 mice (five in each group) including WT (Harlan Laboratories), *C1qa*+/− (10), and *C1qa*−/− treated locally with 15 μL solution of C1q (5 μg) were used in the in vivo experiments. The mice (20 g each) were anesthetized by i.p. injection of zoletil (20 mg/kg)/xylazine (10 mg/kg) (Sigma–Aldrich) and by local treatment with lidocaine (10 mg/kg) (S.A.L.F. Spa). A full-thickness skin sample (5 mm in diameter each) was taken from the shaved dorsum of each animal. Fourteen days later the mice were killed and the skin samples were paraffin-embedded and analyzed by immunohistochemistry for CD34.

Three groups of Wistar rats (three in each group) purchased from Harlan Laboratories were used in the wound-healing experiments. Three skin sample (8 mm in diameter) were taken from the shaved dorsum of each animal (210 g) anesthetized as described above. The ulcers were topically treated with either C1q (5 μg) or VEGF (1 ng) or saline and 14 d later the rats were killed. Paraffin-embedded skin samples were examined for the expression of gC1qR by immunohistochemistry.

The microvascular density was evaluated on CD34-immunosstained sections from rat and mouse skin samples by counting the number of vessels in five microscopic fields at 200x magnification and averaging the counts. Wound size was measured in two dimensions at day 0 and at days 7 and 14 after surgery and the closed wound area was expressed as percentage of the initial punch biopsy area.

All surgical procedures were approved by the Institutional Animal Care Committee of the University of Trieste and performed in accordance with the institutional guidelines and in compliance with the European (86/609/EC) and the Italian (D.L.116/92) laws.

**Statistical Analysis.** Results were expressed as mean ± SD. Statistically significant data in in vitro and in vivo assays were assessed by unpaired two-tailed Student t test. P values of less than 0.05 were considered significant.

Fig. S1. Characterization of the anti-C1r and anti-C1s antibodies. The specificity of the anti-C1r (A) and anti-C1s (B) antibodies was determined by examining the reactivity with normal and C1s-deficient human serum and purified complement components separated by SDS/PAGE under reducing conditions and visualized by Western blot. A: lane 1, NHS (1 μL); lane 2, C1r (1 μg); lane 3, C1s (1 μg); lane 4, C1q (1 μg). B: lanes 1 and 2, C1s (1 and 0.5 μg); lane 3, C1r (1 μg); lane 4, C1q (1 μg); lane 5, NHS (1 μL); lane 6, C1s-deficient serum (1 μL). Note that the upper band in lane 1 corresponds to native C1s and the two lower bands to the activated form of the molecule. Only the latter two bands are visible in lane 2.

Fig. S2. Binding of C1q to HUVEC. ECs grown to confluence on 96-well tissue culture plates were incubated with increasing concentrations of purified C1q for 1 h at room temperature and the bound C1q was revealed by ELISA. The data are presented as mean ± SD of three separate experiments.
Fig. S3. Effect of C1q on HUVEC migration. The cells added to the upper well of a Transwell were allowed to migrate for 12 h toward C1q added in different concentrations to the lower wells. The number of cells migrated into the lower wells was counted by the Coulter counter and expressed as percentage of the total cell number added to the upper well. The data are presented as mean ± SD of three separate experiments.

Fig. S4. Analysis of the combined effect of C1q and VEGF on HUVEC migration. Details of the experimental procedure are given in the legend to Fig S3. Both C1q and VEGF were used at concentrations that exerted a marginal promigratory effect, whereas the mixture of C1q and VEGF induced a significant increase in cell migration compared with C1q and VEGF tested separately. The data are expressed as mean of four independent experiments run in triplicate ± SD; *P < 0.05 vs. control (t test).
Fig. S5. Activation of MAP kinases by C1q and VEGF in HUVEC. EC monolayers were exposed to C1q (10 μg/mL) or VEGF (20 ng/mL) for 15–60 min. After treatment, the cells were lysed and the proteins were separated by SDS/PAGE and analyzed by Western blot using phosphospecific antibodies to recognize the active forms of ERK1/2 and p38 kinase. Untreated cells (0') served as a control. Antibodies to the nonphosphorylated form of ERK1/2 and p38 were used to ensure equal loading of samples. Results are representative of four separate experiments. Densitometric analysis of the bands was performed using ImageJ software and the band intensity was expressed as fold increase in phosphoprotein level relative to untreated cells and normalized to total levels. Data are mean ± SEM from three independent experiments; *P < 0.05, **P < 0.01, ***P < 0.005 (t test).
Fig. S6. Expression of gC1qR on the vascular endothelium of wound and intact rat skin. The presence of the receptor for the globular head of C1q was revealed using a rabbit antibody to human gC1qR cross-reacting with the rat molecule and revealed by the alkaline phosphatase–anti-alkaline phosphatase method with Fast Red chromogen (purple signal). (Scale bars, 30 μm.)

Table S1. Primers used for qPCR and in situ hybridization

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